Mediation of the short-loop negative feedback of luteinizing hormone (LH) on LH-releasing hormone release by melatonininduced inhibition of LH release from the pars tuberalis

(median eminence-pars tuberalis incubation/LH antiserum)

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Contributed by S. M. McCann, May 30, 1991

ABSTRACT The pineal hormone melatonin is thought to mediate the effects of the pineal gland on seasonal reproduction by altering the release of gonadotropins. The mechanism by which melatonin controls gonadotropin secretion has been obscure. Recently, labeled 2-iodomelatonin was used to localize melatonin receptors in brain by radioautography. The highest concentration of melatonin receptors was found in the pars tuberalis of the pituitary gland of mammals. Pituitary hormones, in particular luteinizing hormone (LH), have been localized in cells of the pars tuberalis. Consequently, we hypothesized that melatonin might act on its receptors in the pars tuberalis to alter the release of LH. It would then be possible for this LH to diffuse into the overlying median eminence, there to alter the release of LH-releasing hormone (LHRH) from the axons of the LHRH neurons. To evaluate this hypothesis, we incubated median eminence-pars tuberalis tissue from male rats in vitro. After preincubation in Krebs-Ringer bicarbonate buffer for 30 min, test substances were added to fresh medium and the incubation was continued for 30 min. LHRH or LH released into the medium was measured by radioimmunoassay. Melatonin induced a dose-related release of LHRH with the maximum response at the greatest concentration tested (1 μ M). This concentration of melatonin also significantly reduced the release of LH into the medium. The increased release of LHRH induced by melatonin (10 µM) was completely blocked by the addition of LH (50 ng/ml), which by itself had no significant effect on LHRH release. Rat LH antiserum (final dilution, 1:1800) significantly elevated LHRH output, whereas normal rabbit serum at a similar dilution had no effect. Finally, LHRH $(0.1 \mu M)$ induced a significant release of LH from median eminence-pars tuberalis tissue that was completely blocked by melatonin (10 μ M). The results support the hypothesis that LH released from the pars tuberalis diffuses to the LHRH terminals in the median eminence to suppress LHRH release. Melatonin acts on its receptors in the pars tuberalis to inhibit LH release, thereby stimulating the release of LHRH from its terminals in the median eminence. The negative short-loop feedback of LH inhibits basal LHRH release in vitro since antiserum against LH increased LHRH release. The results suggest a concept concerning the mechanism by which melatonin can affect the release of pituitary hormones from the pars tuberalis. It is likely that these pituitary hormones diffuse into the median eminence to modify the release of hypothalamic releasing and inhibiting peptides, thereby altering plasma pituitary hormone concentrations.

Melatonin, the pineal hormone, responds to seasonal changes in photoperiod to influence reproductive rhythms in seasonal

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breeders; however, the mechanism by which melatonin brings about its diverse effects is unknown (1).

The development of a high-affinity high-specific-activity ligand, 2-[125][iodomelatonin, has allowed the identification of melatonin-binding sites within the brain. The highest concentration of melatonin receptors occurs in the pars tuberalis of mammals (2-4), and it may be that this part of the vertebrate pituitary gland is not the nonfunctional lobe it was thought to be. The cells of this structure have many of the ultrastructural characteristics considered necessary for endocrine secretion (5). Furthermore, seasonal fluctuations in its cytological features, seemingly in concert with reproductive cycles, may represent the morphological expression of seasonal changes in its endocrine function (6).

Luteinizing hormone (LH)-containing cells in the pars tuberalis of rats were first demonstrated through immunocytochemistry by Baker et al. (7), and it is noteworthy that the distribution of these cells corresponds to the distribution of terminals of the LH-releasing hormone (LHRH) neurons in the overlying median eminence. When the median eminence-pars tuberalis was incubated in vitro in the presence of medium with a high potassium concentration, there was release of LH into the medium; LH release could also be stimulated by addition of LHRH (8). Although there is some evidence of LH in hypothalamic neurons, it appears that the largest amount of LH in the median eminence-pars tuberalis complex actually occurs in the gonadotrope-like cells in the pars tuberalis (21).

The concept of short-loop feedback by which pituitary hormones inhibit their own release (9–11) is well established. The short-loop feedback of LH has been thought to be mediated either by LH neurons within the hypothalamus (11) or by retrograde flow of blood in the hypothalamus (11) or by retrograde flow of blood in the hypophyseal portal vessels that transport high concentrations of LH to the median eminence (12). We do not believe that either of these is the major mechanism for the LH negative short-loop feedback. Rather, we hypothesize that LH is released from the gonadotropes of the pars tuberalis and diffuses into the median eminence where it suppresses the release of LHRH from the terminals of the LHRH neurons either by a direct action or by an action of other neuronal terminals.

Melatonin has been shown to exert a dose-related suppression of the forskolin-induced release of cAMP from the pars tuberalis (4). Consequently, we hypothesized that melatonin might act on its receptors on the gonadotropes of the pars tuberalis to lower cAMP that would, in turn, inhibit LH release and remove the LHRH terminals from inhibition, leading to an increase in LHRH release. Consistent with this hypothesis, our *in vitro* results indicate that melatonin does

Abbreviations: LH, luteinizing hormone; LHRH, LH-releasing hormone.

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block the tonic inhibition of LHRH release brought about by pars tuberalis LH.

MATERIALS AND METHODS

Adult male Sprague-Dawley (Holtzmann, Madison, WI) rats (200-250 g) were used. They were housed in group cages under controlled conditions of light (hours lights on, 0500-1900) and temperature (22-25°C, range) and had free access to rat chow and water. The rats were killed between the hours of 1400 and 1600 by decapitation, and their brains were rapidly removed. Under a dissecting microscope, the median eminence-pars tuberalis was dissected from the rest of the brain with fine scissors as described (13). One median eminence-pars tuberalis was placed into a plastic incubation tube containing 350 µl of Krebs-Ringer bicarbonate buffer (KRB). All media contained bacitracin at 0.028 mg/ml to prevent degradation by proteases and were gassed with 95% O₂/5% CO₂ at 37°C. After preincubation for 30 min in a Dubnoff shaker (50 cycles per min), the medium was replaced by fresh medium containing the substances to be tested, and incubation was continued for 30 or 60 min. A sample of medium for LHRH determination was boiled for 5 min and then frozen and stored at -70°C until assayed. Another sample for LH determination was stored at -25°C until assayed. Control and experimental media were subsequently assayed for rat LH using materials provided by the National Institute of Diabetes, Digestive, and Kidney Diseases (NIDDK). LHRH was assayed as described (14) using highly specific LHRH antiserum kindly provided by V. D. Ramirez (University of Illinois, Champagne-Urbana).

To determine the effect of melatonin on LHRH release from the median eminence-pars tuberalis fragments, they were incubated for 30 min with various concentrations of melatonin (10 μ M to 10 nM). Melatonin was initially dissolved in ethanol and diluted to a concentration of 0.05 mM ethanol. Consequently, the control tubes were incubated with an equal volume of the diluted ethanol alone. The effect of melatonin on the release of LH and LHRH into the medium was determined by radioimmunoassay.

To examine the effect of LH on the release of LHRH and its response to melatonin, rat LH was diluted such that the final concentration in the tubes was 50 ng/ml. The tubes containing the median eminence-pars tuberalis fragments were incubated with LH alone or with LH plus melatonin and the effect on release of LHRH was determined.

A possible stimulatory effect of LHRH on release of LH from the median eminence-pars tuberalis tissue was evaluated by incubating the fragments with LHRH at $0.1~\mu M$.

To determine whether LH released in vitro exerts a physiologically significant effect to alter LHRH release by the median eminence tissue fragments, rat LH antiserum was added to the tubes at a final dilution of 1:1800. Control tubes contained an equivalent dilution of normal rabbit serum. The incubation was continued for 1 hr and the LHRH released into the medium was determined by radioimmunoassay.

Melatonin was purchased from Sigma. Rat LH antiserum was kindly donated by A. F. Parlow (Harbor General Hospital, Los Angeles).

Significance of differences between two groups was determined by Student's t test. For multiple comparisons, analysis of variance followed by the Newman–Keuls test was used.

RESULTS

Effect of Melatonin on LHRH Release from Median Eminence-Pars Tuberalis in Vitro. Incubation of median eminence-pars tuberalis samples with melatonin for 30 min induced a dose-dependent stimulation of LHRH release into the medium (Fig. 1), significant at a concentration of $1~\mu M$.

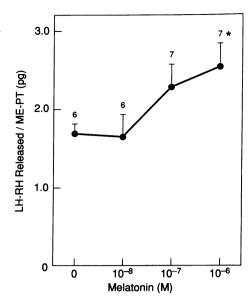


FIG. 1. Effect of various concentrations of melatonin on *in vitro* release of LHRH by median eminence-pars tuberalis (ME-PT) explants. In this and subsequent figures the values are the mean \pm SEM. The number above the data points refers to number of samples. *P < 0.05 vs. control.

This experiment was repeated several times and the minimal effective dose for stimulation ranged from $0.1 \mu M$ to $1 \mu M$.

The Effect of Melatonin on the Release of LH from Median Eminence-Pars Tuberalis in Vitro. Incubation of median eminence-pars tuberalis for 30 min with 1 μ M melatonin significantly decreased the release of LH from median eminence-pars tuberalis explants (Fig. 2).

Blockade by LH of the Stimulation of LHRH Release Induced by Melatonin from Median Eminence-Pars Tuberalis in Vitro. Melatonin (10 μ M) induced significant release of LHRH from median eminence-pars tuberalis explants after incubation for 30 min. The LHRH release stimulated by this concentration of melatonin was completely suppressed in the presence of LH (50 ng/ml). However, LH alone (5 or 50 ng/ml) did not alter basal LHRH release (Fig. 3).

Stimulation of LH Release from Median Eminence-Pars Tuberalis by LHRH and Its Blockade by Melatonin. LHRH $(0.1 \,\mu\text{M})$ induced a highly significant (P < 0.01) release of LH from the median eminence-pars tuberalis, which was completely blocked (P < 0.001) by melatonin $(10 \,\mu\text{M})$ (Fig. 4).

Induction of LHRH Release from Median Eminence-Pars Tuberalis in Vitro by LH Antiserum. Incubation in medium

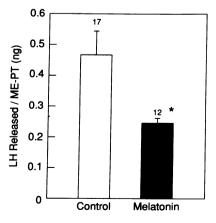


FIG. 2. Inhibition by melatonin (1 μ M) of in vitro basal release of LH by median eminence-pars tuberalis (ME-PT) explants. *P < 0.02 vs. control.

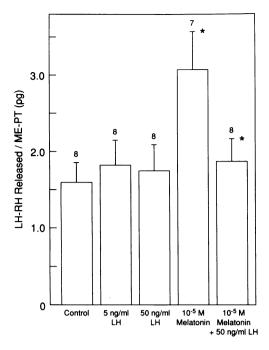


Fig. 3. Effect of LH (50 ng/ml) on *in vitro* basal and melatonin (10 μ M)-induced LHRH release from median eminence-pars tuberalis (ME-PT) explants.

*For 10 μ M melatonin plus LH at 50 ng/ml, P < 0.05 vs. control melatonin-treated group not exposed to LH; for 10 μ M melatonin, P < 0.05 vs. all bars to its left.

containing LH antiserum (1:1800 dilution) for 60 min induced a significant increase of LHRH release from the explants in comparison to the release from control explants incubated in the presence of an equivalent dilution of normal rabbit serum (Fig. 5).

DISCUSSION

The stimulation of LHRH release evoked by melatonin was accompanied by a lowering of the LH content in the medium. This suggests that melatonin acts after combination with its receptors on the gonadotropes of the pars tuberalis to suppress LH release. This action removes an inhibition of LHRH release brought about by basal release of LH from the pars tuberalis. Apparently, this basal release was sufficient to

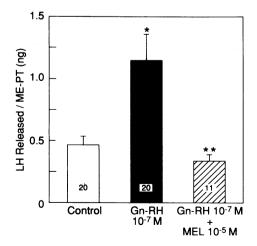


FIG. 4. Effect of LHRH (Gn-RH) on release of LH from median eminence-pars tuberalis (ME-PT) explants and blockade of the effect by melatonin (MEL).

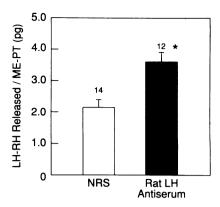


FIG. 5. Effect of rat LH antiserum on *in vitro* release of LHRH by median eminence-pars tuberalis (ME-PT) explants. *P < 0.02 vs. normal rabbit serum (NRS).

cause maximal suppression of LHRH release since the addition of LH alone had no effect on the release of LHRH. The concept that basal LH release suppresses LHRH release was supported by the elevation in LHRH release that occurred in the presence of LH antiserum.

The function of the pars tuberalis has been completely elusive up to this time. Our results support the hypothesis that LH released from pars tuberalis gonadotropes diffuses into the median eminence where it exerts a physiologically significant inhibitory action on the release of LHRH in vitro. Furthermore, melatonin may act on its postulated receptors on these gonadotropes to inhibit adenylate cyclase resulting in a decline in cAMP in the gonadotropes. This could result in subsequent suppression of LH release, which would then decrease the concentration of LH surrounding the terminals of the LHRH axons. This could result in release of the inhibition imposed by LH and, in turn, augmented LHRH release.

This scheme makes it possible to explain the mechanism of the short-loop feedback of LH to suppress its own release, and it also provides a mechanism by which melatonin can augment LHRH release by suppressing the release of LH by the pars tuberalis gonadotropes.

There have been claims for LH-containing neurons in the hypothalamus, in particular in the arcuate nucleus just overlying the median eminence (15); however, it is clear that LH is localized in gonadotrope-like cells of the pars tuberalis (21). This is also the location of the highest concentration of melatonin receptors in the brain and, consequently, we believe that LH in the pars tuberalis is involved in the phenomena described above, although we cannot completely rule out the participation of neuronal LH.

The cells of the pars tuberalis are in intimate contact with neuronal terminals that may contact the individual cells (16). The density of gonadotropes in the pars tuberalis is greatest overlying the lateral median eminence, which also contains the largest numbers of LHRH neuronal terminals (7). Thus, it is possible that LH released from pars tuberalis gonadotropes may alter the release of LHRH from terminals of LHRH neurons abutting directly on these cells.

It is likely that in addition to the feedback of pars tuberalis LH on the median eminence to suppress LHRH release, LHRH released from terminals of LHRH neurons in the median eminence drives the release of LH from the pars tuberalis gonadotropes. Indeed, LHRH-induced stimulation of LH release from the median eminence-pars tuberalis has been demonstrated (8) and confirmed in the present experiments. Thus, LHRH release can drive LH release from the pars tuberalis, which may then diffuse into the median eminence to inhibit further release of LHRH in a negative feedback loop as illustrated in Fig. 6.

^{*}P < 0.01 vs. control.

^{**}P < 0.001 vs. Gn-RH.

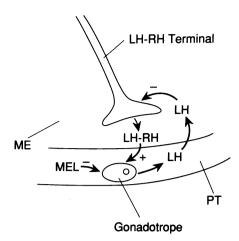


FIG. 6. Summary of feedback loop controlling the secretion of LHRH. MEL, melatonin; ME, median eminence; PT, pars tuberalis; -, suppression; +, release.

It has already been shown that the content of melatonin receptors in the pars tuberalis is under the influence of estrogen; these receptors decline in the castrated animal and are restored by estrogen treatment (17). It may be that these changes in receptors may alter the sensitivity of the effect of melatonin on LHRH release.

In the living animal melatonin generally suppresses LH release and this suppression is thought to result, at least in large part, from suppression of the release of LHRH and yet we have found that *in vitro* melatonin stimulates LHRH release from the median eminence, in agreement with earlier studies (18). It is possible that in the intact animal, melatonin has opposite actions on LHRH release at different sites, the stimulatory action being exerted on the terminals of the LHRH neurons in the region of the median eminence.

Our concept gains support from the fact that there are seasonal changes in pars tuberalis histology associated with altered reproduction during various seasons in seasonal breeders (6) that are thought to be caused by alterations in melatonin release from the pineal gland. Furthermore, during ontogenesis pituitary hormones first appear in the pars tuberalis and only later do they appear in the pars distalis (19).

Since all anterior pituitary hormones are produced by particular cells in the pars tuberalis (20), it is possible that the short-loop negative feedback of all pituitary hormones is mediated by release of the pituitary hormone in question from the pars tuberalis and its diffusion to the median eminence, there to affect the release of the particular releasing or inhibiting hormones in question.

In conclusion, these results open up a vista for understanding the action of the pineal hormone melatonin and the short-loop negative feedback of pituitary hormones to alter their own release.

This work was supported by National Institutes of Health Grants DK10073 and HD09988.

- 1. Reiter, R. J. (1980) Endocr. Rev. 1, 109-131.
- Vanecek, J., Pavlik, A. & Illnerova, H. (1987) Brain Res. 435, 359-363.
- Reppert, S. M., Weaver, D. R., Rivkees, S. A. & Stopa, E. G. (1988) Science 242, 78-80.
- Morgan, P. J., Williams, L. M., Davidson, G., Lawson, W. & Howell, E. (1989) J. Neuroendocrinol. 1, 1-4.
- 5. Fitzgerald, K. T. (1979) Gen. Comp. Endocrinol. 37, 383–399.
- Wittkowski, W., Hewing, M., Hoffman, K., Bergmann, M. & Fechner, J. (1984) Cell Tissue Res. 238, 213-216.
- Baker, B. L., Dermody, W. C. & Reel, J. R. (1975) Endocrinology 97, 125-135.
- Emanuele, N. V., Walloch, L., Baker, G., Kostka, D., Kirsteins, L. & Lawrence, A. M. (1987) Endocr. Res. 13, 251-266
- David, M. A., Fraschini, F. & Martini, L. (1966) Endocrinology 78, 55-60.
- Molitch, M. E., Edmonds, M., Hendrich, J. C., Franchimont, P. & Odell, W. D. (1979) Neuroendocrinology 29, 49-53.
- 11. Conway, S. & McCann, S. M. (1991) Endocr. Res. 16, 403-413.
- Oliver, C., Mical, R. S. & Porter, J. C. (1977) Endocrinology 101, 598-604.
- Negro-Vilar, A., Ojeda, S. R. & McCann, S. M. (1980) in Biochemical Actions of Hormones, ed. Litwack, G. (Academic, New York), Vol. 7, pp. 245-285.
- Dluzen, D. E. & Ramirez, V. D. (1987) Neuroendocrinology 45, 328-332.
- 15. Hostetter, G., Gallo, R. V. & Brownfield, M. S. (1981) Neuroendocrinology 33, 241-245.
- 16. Kotsu, T. & Daikoku, S. (1972) Arch. Histol. Jpn. 34, 167-184.
- 17. Laudon, M. & Zisapel, N. (1987) Brain Res. 402, 146-150.
- Kao, L. W. L. & Weisz, J. (1977) J. Endocrinol. 100, 1723– 1726.
- Nemeskeri, A., Setalo, G. & Halasz, B. (1988) Neuroendocrinology 48, 534-543.
- 20. Ordronneau, P. & Petrusz, P. (1980) Am. J. Anat. 158, 491-506.
- Emanuele, N., Anderson, J., Baker, G., McDonald, D., Kirsteins, L. & Lawrence, A. M. (1984) Brain Res. 309, 271-276.